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(71) Applicant: KABUSHIKI KAISHA FRONTIER [JP/JP]; 518, Waseda Tsurumaki-cho, Shinjuku-ku, Tokyo 169 (JP).

(72) Inventors: NAMIKI, Hideo; Kabushiki Kaisha Frontier, 518, Waseda Tsurumaki-cho, Shinjuku-ku, Tokyo 169 (JP). KURITA, Takeshi; 404-5, Fujima, Kawagoe-shi, Saitama-ken 356 (JP).

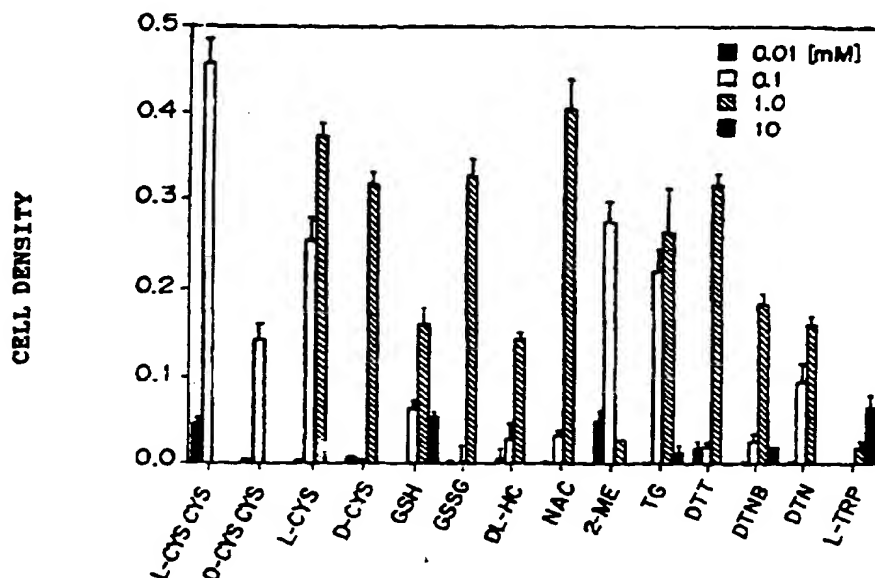
(74) Agents: ISHIKAWA, Yasuo et al.; Park Shiba Building, 4F, 17-11, Shiba 2-chome, Minato-ku, Tokyo 105 (JP).

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(57) Abstract

A first embodiment of a cell culture system has a cell death accelerator comprising one or more cell death inducing substances, including serum albumin, hemoglobin, glycine and glutamic acid. In a second embodiment a cell death inhibitor comprises one or more kinds of cell death inhibiting substances which include mercapto group containing amino acids, other mercapto group containing compounds and tryptophan. In a third embodiment a cell death inhibitor comprises an inhibitor of RNA or protein synthesis, optionally augmented with a thiol. The system can be applied to selectively induce death of cultured cells, such as neoplastic cell lines, or to inhibit death of other cells, such as neoplastic cell lines or non-neoplastic cells such as brain cells.

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CELL DEATH ACCELERATOR AND CELL DEATH INHIBITOR

BACKGROUND OF THE INVENTION

5 1. Field of the Invention.

This invention relates to a cell death accelerator for inducing cell death and a cell death inhibitor for inhibiting cell death.

2. Description of the Related Art.

10 For the purpose of culturing cells in vitro, serum is generally added to a synthetic culture medium in a concentration range of about 10 - 20%. The medium is a pH-balanced salt solution containing various nutrients such as vitamins, amino acids, and sugars. When serum or plasma is highly concentrated in the medium, beyond this range however, cell death is induced.

15 The cell death-inducing activity is seen in all the sera or plasma so far tested regardless of source (species or age), or heat treatment to inactivate complement. Two types of cell death are now generally recognized. The first is passive, resulting from lack of oxygen, mechanical crush injury, or other extreme change in the extracellular environment. The second type of cell death is called apoptosis, an active energy-requiring intracellular process that culminates in
20 DNA fragmentation and osmotic lysis of the cell. The latter is an autonomous physiological death sometimes referred to as programmed cell death, and it occurs normally during development of the nervous system, skin, and other epithelial organs. Morphologically, apoptosis is characterized by blebbing of the plasma membrane and nuclear condensation. These visible changes are
25 accompanied by synthesis of a specific protein(s) including nuclease followed by fragmentation of chromosomal DNA. Several apoptosis-related genes have already been identified in the nematode *C. elegans* and in mammalian lymphocytes.

30 Apoptosis is now the focus of much attention, because it appears to be crucial for normal tissue and organogenesis, neural development, and immune reactions to foreign antigens including those borne by infectious viruses.

The serum contains some components, i.e., proteins such as albumin and globulin, salts such as NaCl, KCl and CaCl₂, vitamins, more than twenty amino acids, such as glycine, cystine, cysteine, alanine and tryptophan, and other components. Heretofore adequate investigation of the individual functions of each component in the serum has not been carried out. The present inventors disclosed that amino acids, such as cystine and cysteine, when added to the serum, decrease the toxic effect of the serum and accelerates growth and/or multiplication of cultured cells in Japanese Patent Application No. 270719/1990 filed on October 9, 1990. At present, however, investigation of the toxic factor itself has not been carried out at all, and the characterization of components which can inhibit the toxicity has been insufficient. In the noted Japanese Application, the inventors disclosed that cysteine and cystine promoted cell growth and/or multiplication, and that tryptophan can prevent cell death.

Recently, Evan et al. (1992) reported that cultured fibroblasts died when the mitotic cycle was interrupted during *c-myc* expression. Serum-induced cell death may involve a similar mechanism. Cells entering the mitotic cycle in response to an excess of growth signals from serum may die after interruption of the cycle by thiol deficiency. Previously, we reported that a cell-death-inducing activity was present in a low molecular weight fraction of serum (M.W. <1,000) (Kurita and Namiki, 1993a). Subsequently, it was found that this activity was inhibited by thiols. It was water-soluble, heat-resistant, and had charcoal affinity. Approximate molecular weight of the factor was approximately 100-200 dalton upon size-sieve HPLC. In addition to low molecular weight factors, traces of macromolecules in the fraction are now thought to be necessary for cell death. The latter may act as a death signal, and *c-myc* may be also involved in the signal transduction. Further studies are needed to address these issues. Nevertheless, serum-induced cell death appears to be a type of apoptosis resulting from a disturbance in thiol metabolism.

SUMMARY OF THE INVENTION

Under the above mentioned circumstances, we have investigated the functions of individual components in the serum, and have achieved the present invention on the basis of the results of the investigation.

It is an object of the present invention to provide a cell death accelerator for inducing cell death.

It is another object of the present invention to provide a cell death inhibitor for inhibiting cell death.

5 According to a first aspect of the invention, a cell death agent comprises at least one cell death inducing substance which is selected from serum albumin, hemoglobin, glycine and glutamic acid to be added to blood. The cell death inducing substances in serum are albumin, hemoglobin, glycine and glutamic acid, and cell death of cultured cells is produced by adding one or more
10 of the above mentioned substances to a culture medium containing serum. For example, the cell death accelerator of the invention can be applied to treat cancer because the cell death inducing substance destroys cancer cells or inhibits growth and/or multiplication of cancer cells.

 According to a second aspect of the invention, a cell death inhibitor
15 comprises at least one cell death inhibiting substance which is selected from mercapto group containing amino acids such as cysteine, cystine and so forth, other mercapto group containing compounds and tryptophan, the selected substance to be added to blood.

 The cell death inhibiting substances in the serum are mercapto group
20 containing amino acids, other mercapto group containing compounds and tryptophan. When one or more of the above mentioned cell death inhibitors are added to a culture medium containing serum, the inhibitors counteract the toxic effects of the toxic factor in the serum, and, thereby the cell death of cultured cells is inhibited. For example, if the cell death inhibiting substances are added
25 to an instillation injection, and then injected into the central nervous system of a patient, and directly supplied to a diseased part during treatment of brain apoplexy, eye ground apoplexy and so forth, death of the patient's cells can be prevented efficiently.

 We had previously found that various sera supplemented with amino
30 acids and vitamins in quantities equivalent to those in basal tissue culture medium inhibited cell growth but did not induce cell death. Herein we disclose the identity of the rescue factors as L-cyst(e)ine and L-tryptophan, and we

examine the effects of thiol-containing molecules other than L-cysteine on serum induced cell death. All of them were protective in varying degrees. Death is also prevented by several inhibitors of protein and RNA synthesis. Interestingly, N-acetyl-L-cysteine (NAC) was as protective as other thiols, but it did not inhibit uptake of L-[³⁵S]methionine, suggesting that thiol bearing molecules were not acting as inhibitors of protein or RNA synthesis. Treatment of cells cultured in FBS with NAC prevented a reduction in their thiol content, but protein and RNA synthesis inhibitors had no corresponding effect. In addition, we also demonstrated DNA fragmentation prior to the breakdown of the plasma membrane. These findings suggest that serum-induced cell death represents a thiol-mediated apoptosis.

The invention provides a cell system includes a concentration of a low molecular weight fraction of serum. A population of living cells are exposed to the serum, and optionally to a concentrated and refined molecular species that prevents induction of death of the cells by whole serum. The cells can be human fetal lung fibroblasts, human epithelioid carcinoma cells, or mouse melanoma cells.

According to an aspect of the invention the molecular species is a thiol or a dithiol homo-dimer thereof. The molecular species can be at least one of the group consisting of cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, dithionite, thioglycolic acid, DL-homocystine, N-acetyl-L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid), a homo-dimer thereof, and a mixed disulfide thereof.

Preferably the molecular species has a concentration of between about 1 and 10 mM.

The invention provides a cell system which includes a concentration of serum. A population of living cells are exposed to the serum, for example fetal bovine serum, and to a concentrated and refined molecular species that accelerates induction of death of the cells. The molecular species is selected from the group consisting of serum albumin, hemoglobin, glycine and glutamic acid. The cells can be human fetal lung fibroblasts, human epithelioid carcinoma cells, or mouse melanoma cells.

The invention provides a method of preventing cell death in a cell system which is accomplished by providing a concentration of a low molecular weight fraction of serum, which is equivalent to a cytotoxic concentration of whole serum, the latter being sufficient to induce cell death, exposing a population of living cells to the low molecular weight serum fraction, and optionally exposing the living cells to a concentrated and refined molecular species that prevents induction of death of the cells by the serum. The cells can be human fetal lung fibroblasts, human epithelioid carcinoma cells, or mouse melanoma cells. According to an aspect of the invention the molecular species is a thiol or a dithiol homo-dimer thereof. The molecular species can be at least one of the group consisting of cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, dithionite, thioglycolic acid, DL-homocystine, N-acetyl-L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid), a homo-dimer thereof, and a mixed disulfide thereof.

Preferably the molecular species has a concentration of between about 1 and 10 mM.

The invention provides a method of preventing serum-induced apoptotic cell death, which is accomplished by providing a cell culture medium which has a concentration of a low molecular weight fraction of serum, which is equivalent to a cytotoxic concentration of whole serum, the latter being sufficient to induce cell death, enriching the medium with an amino acid selected from the group consisting of L-cysteine, L-cystine, and L-tryptophan, and culturing a cell line in the enriched medium.

According to an aspect of the invention the amino acid is L-cysteine in a concentration of between about 0.1 mM and 5 mM.

In another aspect of the invention the amino acid is L-cystine in a concentration of between about 0.05 mM and 0.5 mM.

The invention provides a method of preventing serum-induced apoptotic cell death, which is performed by providing a cell culture medium has a cytotoxic concentration of serum, enriching the medium with a substance selected from the group consisting of puromycin hydrochloride, emetine hydrochloride, cycloheximide, actinomycin D, ethidium bromide, L-tryptophan, and D-

tryptophan, and culturing a cell line in the enriched medium.

According to an aspect of the invention the medium is enriched with a thiol, which can be N-acetyl-cysteine.

The invention provides a method of preventing serum-induced
5 apoptotic cell death, which is performed by providing a cell culture medium having a cytotoxic concentration of serum, culturing a cell line in the enriched medium, and while the cells are growing in culture, inhibiting protein synthesis or RNA synthesis in the cell line.

BRIEF DESCRIPTION OF THE DRAWING

10 For a better understanding of these and other objects of the present invention, reference is made to the detailed description of the invention, by way of example, which is to be read in conjunction with the following drawings, wherein:

FIG. 1 is a chart showing the cell death inhibiting effects of substances
15 on cultured cells;

FIG. 2 is a chart showing the cell death inducing effects of substances on cultured cells;

FIG. 3 is a chart indicating the effect of serum concentration on cell growth;

20 FIG. 4 is a chart illustrating rescue from serum-induced cell death by amino acids;

FIG. 5 illustrates rescue from serum-induced cell death by inhibitors of protein and RNA synthesis;

25 FIG. 6 is a chart illustrating the effect of protein and RNA synthesis inhibitors on methionine uptake in cultured cells;

FIGS. 7a and 7b are charts respectively illustrating total thiol content and ethanol-soluble thiol content of cultured cells following incubation in the presence of various reagents;

30 FIG. 8 is a chart illustrating the relationship of live cultured cells to time in an experiment on serum toxicity; and

FIGS. 9a and 9b are schematic illustrations of two models of serum-induced cell death.

DESCRIPTION OF THE PREFERRED EMBODIMENT**Abbreviations**

As used herein certain abbreviations and acronyms have the following meanings:

	ACT D	actinomycin D	FBS	fetal bovine serum
5	CHX	cycloheximide	GSH	glutathione reduced
	CYS	cysteine	GSSG	glutathione oxidized
	CYS-CYS	cystine	2-ME	2-mercaptoethanol
	DL-HC	DL-homocystine	NAC	N-acetyl-L-cysteine
	DTN	sodium dithionite	NON	non-inhibitor
10	DTNB	5,5'dithiobis(2-nitrobenzoic acid)	PBS	Phosphate buffered saline
			PMC	puromycin.2HCl
	DTT	dithiothreitol	TGA	thioglycolic acid
	EAA	essential amino acids	MEM	minimal essential medium
	EMT	emetine.2HCl	NEAA	nonessential amino acids
15	ET Br	ethidium bromide		

In one experiment, a cell system had a cytotoxic concentration of serum sufficient to induce cell death. A population of living cells was exposed to the serum, and a concentrated and refined molecular species that prevents induction of death of the cells by the serum was included in a culture medium. Suitable cells were human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells. More particularly TIG-cells, i.e., human fetal lung fibroblasts, were seeded into a 96-well multiplate (1×10^4 cells/well) and incubated overnight. Thereafter the culture medium was removed to be exchanged with test medium, a culture medium containing fetal bovine serum (FBS) and various selected reagents. Six days after the exchange of the culture medium, living cells in the culture medium were counted using a dye elution method to obtain the living cell density associated with each reagent.

The molecular species used was a thiol or a dithiol homo-dimer thereof, or a mixed disulfide in various concentrations which included the range of between about 1 and 10 mM.

The following reagents were used: L-cystine (L-CYSCYS); D-cystine(D-CYSCYS); L-cysteine (L-CYS); D-cysteine (D-CYS); reduced glutathione (GSH); oxidized glutathione (GSSG); DL-homocystine (DL-HC); N-acetyl-L-cysteine (NAC); 2-mercaptoethanol (2-ME); thioglycolic acid (TG); dithiothreitol (DTT);
5 5,5'-dithiobis(2-nitro benzoic acid) (DTNB); sodium dithionite (DTN); and L-tryptophan (L-TRP). The concentration of each reagent was set at four levels: 0.01 mM, 0.1 mM, 1.0 mM and 10 mM.

A result of the experience is shown in FIG. 1. In FIG. 1, each reagent is indicated along the abscissa and the corresponding cell density is indicated
10 along the ordinate. The height of each column in the graph indicates the mean of the measured cell densities of six wells, and a bar on the top of each column, if shown, indicates the standard error.

According to the result, reagents which had a mercapto group in their chemical structures conduced to inhibition of the cell death. L-CYSCYS, D-
15 CYSCYS, L-CYS, 2-ME, TG and DTN showed greatly inhibiting effects. They could prevent cell death even at a concentration of 1 mM. It was recognized that mercapto group containing amino acids and other mercapto group containing compounds could inhibit cell death.

Experiments on cell death inducing substances which exist in serum will
20 now be explained. A cell system was used which had a cytotoxic concentration of serum sufficient to induce cell death. A population of living cells was exposed to the serum, and the culture medium contained a concentrated and refined molecular species that accelerates induction of death of the cells by the serum. The molecular species was one of the group consisting of serum albumin,
25 hemoglobin, glycine and glutamic acid. Suitable cells were human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells.

A low molecular weight fraction in FBS serum was freeze-dried, and a solvent then added. The insoluble fraction in was removed by centrifugation to purify the low molecular weight fraction. The soluble fraction thereby obtained
30 was freeze-dried again, and washed in acetonitrile. The resulting freeze-dried substance was dissolved in distilled water to prepare a sample solution having an approximately 20-fold dilution of the low molecular weight fraction with respect

to FBS, supposing that recovery of the low molecular weight fraction therefrom is 100%. Thereafter, the sample solution was filtered through a filter having a 0.2 μ mesh.

5 This sample solution was added to Hank's solution so as to obtain a test medium containing 10 volume % of above mentioned sample solution, 1 volume % of FBS and 10 volume % of MEM. Further, each reagent or combination of the reagent and cysteine being tested was added to the test medium at reagent concentrations of 1 mM and 10 mM.

10 TIG-cells were seeded into a 24-well multiplate (5×10^3 cells/well) having the test medium and incubated for 48 hours. Thereafter living cell density was measured using the dye elution method.

According to the experimental results, glycine, glutamic acid and tryptophan showed a cell death inducing effect at 1 mM. Phenylalanine, aspartic acid, asparagine and glutamine also showed a cell death inducing effect at 10 mM. On the other side, tyrosin, serine and glycyglycine did not show a cell death inducing effect, but inhibited growth and/or multiplication of cells. It was furthermore proved that hemoglobin had growth and/or multiplication inhibiting effect in the same manner.

20 These and other experiments will now be disclosed in further detail.
Cell cultures

Human fetal lung fibroblasts (TIG-1), human epithelioid carcinoma (HeLa) cells, and mouse melanoma (B16) cells were obtained from the Japanese Cancer Research Resources Bank. All cells were maintained in Eagle's MEM containing 10% FBS at 37°C. Cell cultures were examined and photographed with a Nikon Diaphot phase-contrast inverted microscope.

Reagents

Amino acids: L-cyst(e)ine (Kanto Chemical), D-cysteine (Sigma), D-cystine (Wako), L-tryptophan (Kanto), D-tryptophan, DL-homocystine (Wako), glutathione oxidized form (Sigma grade III), reduced form (Merck), dithiothreitol (Wako), 2-mercaptoethanol, thioglycolic acid, N-acetyl-L-cysteine, sodium dithionite (Kanto), 5,5'-dithiobis(2-nitro benzoic acid) (Kanto), emetine.2HCl

(Fluka AG.), cycloheximide, puromycin. 2HCl, actinomycin D, ethidium bromide (Sigma).

Serum and media

FBS was from Boehringer Mannheim (lot. 614413, 562044, 147013).

- 5 Eagle's MEM "Nissui" was from Nissui Pharmaceutical Co., EAA (essential amino acids) supplement for MEM x 50, NEAA (nonessential amino acids) supplement for MEM x 100 and vitamin supplement for MEM x 100 were from Boehringer Mannheim, Flow Laboratories, and Dainippon Pharmaceutical Co., respectively.

Ultrafiltration of FBS

- 10 FBS was filtered through an ultrafiltration membrane YM2 (M.W.1,000) (Amicon Co.). FBS was concentrated tenfold and diafiltered with a tenfold volume of deionized water to remove the low-molecular-weight fraction. The resultant macromolecular fraction of FBS was again concentrated tenfold and was diluted to the original FBS volume with 10/9 concentrated Eagle's MEM. pH
15 and osmolality were adjusted to 7.2 ± 0.2 and 290 ± 10 mosmol/Kg.H₂O), as the low-molecular-weight-fraction depleted FBS.

Automated cell counting

- Appropriate numbers of cells were seeded into 96-well culture plates containing Eagle's MEM, 10% FBS (100 μ l/well) (Corning). After overnight
20 incubation at 37°C in 5% CO₂, the culture medium was removed. Wells were washed with calcium and magnesium-free PBS (CMF-PBS), and 100 μ l of test medium was added. After several days of incubation, cells were harvested by trypsinization and suspended in Isotone II (Coulter Electronics). Cell number was measured with a Coulter Counter ZM (Coulter Electronics) and displayed
25 numbers were corrected with standard hemocytes. We previously verified that the numbers displayed on the counter were in agreement with those counted using a hemocytometer. Results were expressed as the means (\pm SE) of six independent measurements.

Cell counting by dye elution method

- 30 TIG-1 cells were seeded in 96-well microplates in 100 μ l of Eagle's MEM, 10% FBS. After several day's incubation, medium was removed and the cells were washed with CMF-PBS. Cell quantity was determined by the method

of Hori et al. (1988) with a minor modification. Cells were stained with 50 μ l of 0.5% crystal violet per well in ethanol/water (1:4) for 10 min and then washed with water. The dye was eluted with 150 μ l of 33% acetic acid per well, and the absorbance at 600 nm was measured by a microplate reader (CORONA MTP-22). Results were expressed as the means (\pm SE) of six independent determinations. We preliminarily confirmed that the absorbance at 600 nm was proportional to the density of TIG-1 cells cultured in MEM, 10% FBS.

Cell viability

TIG-1 cells were seeded in 48-well microplates in 100 μ l of Eagle's MEM, 10% FBS. After incubation, cells were harvested with 0.1% trypsin, in CMF-PBS, and 0.5% trypan blue in PBS was added. Cells that were not stained were counted with a hemocytometer. Results were expressed as the means (\pm SE) of four independent determinations.

L-[35 S]methionine uptake

TIG-1 cells were seeded into a 24-well multiplate at 1/2 confluency (about $\times 10^5$ cells/well) and incubated overnight. The next morning, the culture medium was removed and changed to test medium (20 μ Ci L-[35 S]methionine (Amersham), 20% CMF-PBS, 80% FBS). After appropriate time (0-16 hr) of incubation, each culture was washed three times with 0.02% EDTA CMF-PBS, and the cells were lysed with 1% SDS. Macromolecules were precipitated with each 10 μ l TCA and centrifuged, and the precipitate was washed with 10% TCA. Then, it was dissolved in 300 μ l of 1% SDS, 100 μ l of which was dissolved in 1 ml of liquid scintillation cocktail and counted (Aloka LSC-700).

Thiol content assays

TIG-1 cells were cultured in a 24-well plate (3×10^5 cells/well) in fresh medium for 24 hr. The medium was then changed to the test medium, MEM plus 10% FBS, Hank's balanced solution, or whole FBS supplemented with various reagents. Total thiol content was determined as follows. After appropriate hr (0-16) of incubation, cells were washed twice with 0.02% EDTA, CMF-PBS, and they were lysed with 200 μ l/well of 1% SDS, 0.02% EDTA, 50 mM Tris-HCl (pH.8.2). The lysate was dyed with 10 μ l of 20 mM DTNB dissolved in methanol. Pre-chilled ethanol (600 μ l) was added to precipitate the macromolecules,

and precipitates were collected by centrifugation at 13,000xg for 20 min. Absorbance at 415 nm of the supernatant was measured by a microplate reader (Bio Rad 3550). Concentration of thiol was calculated by comparison to a GSH standard. Ethanol-soluble thiol was measured as follows. After appropriate hr
5 (0-16) of incubation, cells were washed twice with 0.02% EDTA, CMF-PBS, and they were lysed with 100 μ l/well of 1% SDS, 0.02% EDTA, 50 mM Tris-HCl (pH.8.2). Macromolecules were precipitated with 300 μ l of pre-chilled ethanol, and precipitates were removed by centrifugation at 13,000xg for 20 min. The supernatant was dyed with 10 μ l of 20 mM DTNB dissolved in methanol, and the
10 absorbance at 415 nm of the supernatant was measured by a microplate reader. Concentration of thiol was calculated by comparison to a GSH standard. Thiol content was expressed as SH mol/living cell number.

DNA fragmentation assay

TIG-1 cells (3.5×10^5 cells/35 mm dish) were washed twice with CMF-PBS and lysed in 0.8 ml 0.6% TE buffer with 1 μ g/ml Rnase. Then, 200 μ l of 5
15 M NaCl was added to the solution and stored at 4°C overnight, and macromolecular DNA was pelleted by centrifugation at 13,000xg for 30 min. DNA in the supernatant was purified by phenol-chloroform extraction, and traces of phenol were removed by extracting twice with chloroform. Purified DNA was collected
20 by centrifugation at 13,000xg for 20 min with 3 M potassium acetate and 70% ethanol. Precipitated DNA was dissolved in TE buffer, and 1/5 to 1/3 volume of the solution was electrophoresed in a 2.6% agarose gel. Gel was stained by 0.5 μ g/ml ethidium bromide and photographed in 254 nm ultraviolet.

We previously discovered that most types of cultured cells died in
25 concentrations of serum exceeding 60%. However, low molecular weight fraction-depleted serum whose osmotic pressure was adjusted with Eagle's MEM (UF-FBS) never showed cytotoxicity. The low molecular weight fraction thus filtered was similar to MEM in salt content, but it was deficient in nutrients such as vitamins and amino acids. We therefore enriched FBS with these nutrients
30 equivalent to the amounts present in MEM for cell culture.

FIG. 3 shows the effect of serum concentration on TIG-1 cell growth, wherein \square indicates Unenriched FBS; \blacktriangle indicates FBS + AA VIT; and \circ indicates

low molecular weight fraction-depleted FBS. In developing the data illustrated in FIG. 3, TIG-1 cells were seeded at a density of 5×10^3 cells/well in 96-well microplates in Eagle's MEM containing 10% FBS. After overnight incubation, medium was replaced with Eagle's MEM containing several concentrations of FBS. After 6 days, cells harvested by trypsinization were counted with a Coulter counter.

As shown in FIG. 3, cells died at a high concentration of unenriched FBS (Whole FBS), but not in the UF-FBS supplemented with MEM. In addition, cell growth was suppressed in enriched FBS as the concentration was increased. Nevertheless, the cells remained viable, suggesting MEM contained molecules that prevented cell death. Among the vitamins and amino acids tested, only the latter showed this rescue effect. One half to fourfold MEM equivalent of amino acids rescued TIG-1 cells from serum-induced cell death (data not shown). We measured the concentration of each amino acid in FBS. Among essential amino acids, cyst(e)ine and arginine were of less concentration in FBS as compared to those in MEM, which were 8-15 vs. 24 and 1-6 vs. 126, respectively. To identify the amino acids responsible for the rescue effect, each of the 20 L-amino acids involved in protein synthesis and L-hydroxyproline were tested (data not shown).

Rescue from serum-induced cell death by amino acids is illustrated in FIG. 4. In developing the data illustrated in FIG. 4 TIG-1 cells were seeded in 96-well microplates at a density of 5×10^3 cells/well in Eagle's MEM containing 10% FBS. After overnight incubation, medium was replaced with FBS containing amino acids. After 6 days, cells were counted with a Coulter counter. Only two amino acids, L-cyst(e)ine and L-tryptophan, showed ability to rescue cells from serum-induced toxicity (FIG. 3) With L-cysteine, cell death was completely prevented at 0.1 mM, and cells grew well at 1 mM. With L-cystine, whose maximum solubility is ~0.5 mM, protective effect were observed beginning at 0.05 mM (0.1 mM cysteine equivalent). In contrast, L-tryptophan was effective only at concentrations above 5 mM, and it only partially rescued the cells. Concentrations of L-cysteine higher than 5 mM sometimes inhibited cell growth. Similar to TIG-1 cells, HeLa cells and B16 cells were also protected by L-cyst(e)ine and L-tryptophan (data not shown).

The rate of glutathione synthesis in cultured fibroblasts is known to depend on the cystine content of the medium (Meister and Tate, 1976), and when glutathione was added to the medium of TIG-1 cells, death was prevented. This also occurred when oxidized glutathione (GSSG) was tested. There was no evidence of extracellular enzymatic reduction of GSSG or its transport into the cell. Accordingly, the rescue activity of glutathione may not be due to its ability to conjugate to other molecules via thiol linkages.

To further study the roles of cysteine and glutathione, experiments were performed as before using reducing agents, dithiols, L-amino acids, denaturants, and an active oxygen-eliminating enzyme. These reagents are listed below. Reducing agents: L-ascorbic acid, L-ascorbic acid phosphate, Mg, DL- α -tocopherol, dithiothreitol, 2-mercaptoethanol, thioglycolic acid, N-acetyl-L-cysteine, sodium dithionite, potassium ferrocyanide, thiourea. Dithiols: DL-homocysteine, 5,5'-dithiobis(2-nitro benzoic acid); L-amino acids: L-tryptophan, L-methionine; chelates: ethylene diamine tetra-acetic acid and ethylene glycol bis(2-aminoethylether). Denaturants: urea, sodium dodecyl sulfate; Active-oxygen eliminating enzyme: bovine erythrocyte superoxide dismutase (SOD) (WAKO).

FIG. 1. illustrates rescue from serum-induced cell death by thiols. In developing the data shown in FIG. 1, TIG-1 cells were seeded in 96-well microplates at a density of 1×10^4 cells/well in Eagle's MEM containing 10% FBS. After overnight incubation, medium was removed, and 80 μ l of FBS plus 20 μ l of PBS containing cysteine (CYS), cystine (CYS-CYS), glutathione oxidized (GSSG), glutathione reduced (GSH), dithiothreitol (DTT), 2-mercaptoethanol (2-ME), sodium dithionite (DTN), thioglycolic acid (TGA), DL-homocysteine (DL-HC), N-acetyl-L-cysteine (NAC), or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were added to each well. All reagents were tested for rescue from cell death at concentrations ranging from 10 μ M to 1 mM, in some cases to 10 mM. D- and L-cystine were tested in the range of 1 μ M to 100 μ M, because of low solubility. After 4 days, cell number was determined by the dye elution method. Reagents were tested in concentrations from 1 μ M to 1 mM or 1 μ M to 10 mM, except in the case of SOD, which was tested in the range 10^1 - 10^4 units/ml. Only rescue activity-positive data from independent experiments are

depicted in FIG. 1. All reagents bearing SH or cleaved dithiols possessed the rescue activity, and cysteamine and cystamine also were effective in preventing cell death. In contrast, the nonthiol-reducing agents were inactive (data not shown).

5 To determine whether death was due to apoptosis or necrosis, various inhibitors of protein synthesis and RNA synthesis were tested for the rescue activity, since it is generally accepted that in apoptosis specific protein(s) are synthesized. The procedure is explained with reference to FIG. 5. which illustrates rescue from serum-induced cell death by inhibitors of protein and RNA
10 synthesis. The ordinate in FIG. 5 represents the number of viable TIG-1 cells after 24 hr incubation. Open columns indicate reagent only. Striped columns indicate reagent + 1 mM N-acetyl-L-cysteine (NAC). NON, PBS only; CHX, 1 μ g/m1 cycloheximide; PMC, 1 μ g/m1 puromycin.2HCl; EMT, 1 μ g/m1 eme-
15 μ g/m1 actinomycin D; ET Br, 25 μ g/m1 ethidium bromide. TIG-1 cells were seeded in 48-well microplates at a density of 3×10^4 cells/well in Eagle's MEM containing 10% FBS. After 3 days incubation, medium was replaced with 200 μ l of FBS plus 50 μ l of PBS containing each reagent. After 24 hr, the number of viable cells was determined by a hemocytometer. All the reagents were tested
20 for rescue from cell death at concentrations ranging from 0.1 μ g/m1 to 10 μ g/m1 except for D- and L-tryptophan. Data at the most effective concentration are shown in this graph. All reagents were tested at concentrations ranging from 0.01 to 10 μ g/m1, and in a separate group 1 mM NAC was added as a positive control. Protein synthesis inhibitors CHX, PMC and EMT, and RNA synthesis
25 inhibitors ACT D and ET Br, prevented death of cells in these cultures, suggesting that serum-induced toxicity requires protein synthesis.

Inhibitors of protein and RNA synthesis protected cells against serum-induced toxicity similar to thiols, yet they never showed a growth-promoting effect. Accordingly, these inhibitors may act via a molecular mechanism different
30 from that of thiols. To determine whether thiols inhibited protein synthesis, L-[35 S]methionine uptake into TIG-1 cultured in FBS was measured. A typical result from three independent experiments is presented in FIG. 6. To develop

the data shown in FIG. 6 TIG-1 cells were seeded into a 24 well-multiplate at a half-confluency (about 6×10^4 cells/well), and medium was replaced with FBS containing 20 μ Ci L-[35 S]methionine. After incubation for each indicated time, cells were lysed and macromolecules were precipitated with TCA. Precipitates were dissolved in buffer and counted by liquid scintillation. TIG-1 cells cultured in FBS showed substantial uptake of L-[35 S]methionine, suggesting that cells vigorously synthesized protein prior to death. L-[35 S]methionine uptake into TIG-1 cells in all three experiments was completely inhibited by 1 μ g/ml CHX and partially inhibited by 5 mM L-tryptophan (30-50%). By comparison, 1 mM NAC did not inhibit L-[35 S]methionine uptake.

We interpreted these results to indicate that supplemental thiols prevent cell death by maintaining intracellular thiol levels. We found, however, that acid-soluble thiol content of TIG-1 cells cultured in FBS quickly decreased whether or not the medium was supplemented with thiol (data not shown). Thereafter, total and ethanol-soluble thiol content were measured in subsequent experiments. As shown in FIGS. 7a and 7b, both thiol contents were conservative when 1 mM NAC was added to FBS, whereas protein synthesis inhibitor CHX did not affect thiol content either total and ethanol-soluble. The following procedure was followed in developing the data plotted in FIGS. 7a and 7b: After several hours incubation in test medium, cells were lysed in 1% SDS, 0.02% EDTA, 50 mM Tris-HCl (pH.8.2). Either the total lysate or the lysate after precipitation of macromolecules by x3 volume of ethanol (ethanol-soluble) was dyed with 20 mM DTNB. Absorbance at 415 nm was measured by microplate reader 3550 (Bio Rad). Concentration of thiol was calculated by comparison to a GSH standard. Thiol content was expressed as SH mol/living cell number. FIGS. 7a and 7b are to be interpreted in conjunction with the following key:

- | | | | |
|---|---------------------------------|---|--------------------|
| ● | 10% FBS containing Eagle's MEM; | ▲ | FBS + 1 mM NAC; |
| □ | Hank's balanced solution; | ○ | 1 μ g/ml CHX; |
| △ | FBS; | ■ | FBS + 10 mM L-TRP. |

Another distinguishing feature of apoptosis, namely, DNA fragmentation, was evaluated by agarose gel electrophoresis of low molecular weight DNA from dying cells at various stages of serum toxicity. Living cell ratios were measured by the method of trypan blue exclusion, as illustrated in FIG. 8. The chart shown in FIG. 8 reflects the result of the following procedure: TIG-1 cells were seeded into a 35 mm culture dish at a density of 2×10^5 cells/dish. When the density reached 3.5×10^5 cells/dish, medium was replaced with FBS. After the incubation for each indicated time, cells were harvested with 0.1% trypsin in CMF-PBS, and 0.5% trypan blue in PBS was added. Cells that excluded the dyed were counted as living with a hemocytometer. Results are expressed as the means (\pm SE) of four independent determinations. Until 6 hr of the serum treatment, the cells appeared morphologically viable and were not stained by trypan blue. At that point, viability declined steeply, and all cells were dead by 12 hr. DNA fragments (ladder) appeared at 6 hr of the treatment before the plasma membrane breakdown, and they persisted until 12 hr. Electrophoresis of DNA fragments of dying cells (not shown) was conducted using 2.6% agarose-gel. The maximal length of the DNA ladder was relatively small (<0.7 kbp), and the space between rungs of the ladder was slightly smaller than the 0.2 kbp value reported for the DNA ladder usually observed during apoptosis (Kerr et al., 1972; Wyllie et al., 1980; Ellis et al., 1991; Raff, 1992; Eastman, 1993). Nevertheless, DNA fragmentation proceeded morphological change by a few hr, and RNA and protein synthesis inhibitors prevented cell death, consistent with the view that serum-induced toxicity is a type of apoptosis.

In the present study, we observed that serum-induced toxicity was prevented with thiol (SH)-related molecules. Disulfide, D-cyst(e)ine, and DTNB were effective in rescuing cells despite the fact that these compounds are not normally present in cells and do not function as reducing agents in medium. In addition, dithionite, a reducing disulfide, was protective. The findings suggest that these reagents may promote efficient cellular utilization of SH by dithiol exchange or by a reaction involving chemical reduction. The standard cell culture system is an oxidative environment, where SH is oxidized to form dithiol. SH-compounds like cysteine and glutathione in serum are, therefore, able to

form dithiol homo-dimers or mixed disulfides with low molecular weight thiols and proteins. In contrast, cells find little use for mixed disulfides. However, if molecules such as L-cysteine, reduced glutathione, or L-cystine are produced through exchange or reducing, they may be readily used by cells, resulting in promotion of intracellular SH metabolism.

Intracellular thiol levels were decreased in high concentrations of serum, and addition of thiols reversed the decline. Thiol content began to decrease several hours prior to plasma membrane breakdown, and this suggests that the lowering of thiol content was a causal factor in serum-induced cell death rather than a result. It was not, however, the only cause of death, because Hank's balanced solution did not induce death even though thiol content was reduced as in the case of cells cultured in FBS. Protein synthesis inhibitors prevented cell death, but they had no effect on intracellular thiol content, suggesting that protein synthesis was necessary for serum-induced toxicity.

Taken as a whole, the evidence is consistent with two hypotheses for serum-induced cell death, schematically illustrated in FIGS. 9a and 9b. The first is that a decrease in thiol content directly induces synthesis of proteins required for programmed cell death (FIG. 9a). The second is that a decrease in thiol content affects a variety of intracellular processes that occur during cell death including protein synthesis (FIG. 9b). Whether either of these hypotheses can be experimentally verified remains to be determined. During protein synthesis, de novo RNA synthesis may not be necessary, since RNA synthesis inhibitors only partially protected against death of HeLa cells and low density cultures of TIG-1 cells. High concentrations of L- and D-tryptophan showed equal ability to rescue cells. To our knowledge, inhibition of protein synthesis by tryptophan has not been reported, yet our preliminary study showed that L-tryptophan incompletely inhibited L-[35S]methionine uptake by cultured TIG-1 cells (FIG. 6). This may indicate that L- and D-tryptophan can act as protein synthesis inhibitors in some cases.

Addition of exogenous thiol compounds maintained the intracellular level of total thiol, including the protein fraction against depletion by serum-induced toxicity. The level of acid-soluble thiol was, however, not always

restored by the addition, whereas that of ethanol soluble thiol moderately increased. Ethanol is known to solubilize short peptides more efficiently than TCA, and this may be the reason why the ethanol soluble thiol level was higher. These results suggest that supplemental thiol was predominantly incorporated into protein fractions that maintained the total thiol level.

It is possible that thiols contained in protein fractions may act as modifiers of cysteine residues. For example, some enzymes require modification of their cysteine residues by glutathione for activation (Ziegler, 1985). Serum-induced toxicity may reflect such a functional disorder caused by a shortage of thiols.

A second major observation was that DNA was cleaved into fragments beginning a few hours before plasma membrane breakdown. The electrophoretic behavior of the DNA differed from ordinary apoptosis in that the fragments were smaller, and sometimes the DNA appeared to be randomly digested, showing a smeared pattern rather than discrete bands. Nevertheless, a characteristic feature of apoptosis was evident, namely, DNA fragmentation prior to membrane disintegration.

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While this invention has been explained with reference to the structure disclosed herein, it is not confined to the details set forth and this application is
20 intended to cover any modifications and changes as may come within the scope of the following claims:

CLAIMS

1. A cell system comprising:
 - a concentration of a low molecular weight fraction of a serum, said concentration being at least equivalent to a cytotoxic concentration of whole serum sufficient to induce cell death;
 - a molecular species that prevents induction of death of said cells by said whole serum; and
 - a population of living cells that are exposed to said low molecular weight fraction and said molecular species.
2. The system according to claim 1, wherein said cells are selected from the group consisting of human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells.
3. The system according to claim 1, wherein said fraction comprises a fraction of fetal bovine serum and said whole serum comprises whole fetal bovine serum.
4. The system according to claim 1, wherein said molecular species comprises a thiol or a dithiol homo-dimer thereof.
5. The system according to claim 1, wherein said molecular species comprises at least one of the group consisting of cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, dithionite, thioglycolic acid, DL-homocystine, N-acetyl-L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid), a homo-dimer thereof, and a mixed disulfide thereof.
6. The system according to claim 5, wherein said molecular species has a concentration of between about 1 and 10 mM.
7. A cell system comprising:

a low molecular weight fraction of serum;
a population of living cells that are exposed to said serum; and
a molecular species that accelerates induction of death of said cells,
wherein said molecular species is selected from the group consisting of serum albumin, hemoglobin, glycine and glutamic acid.

8. The system according to claim 7, wherein said cells are selected from the group consisting of human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells.

9. The system according to claim 7, wherein said serum comprises fetal bovine serum.

10. A cell system comprising:
a serum;
a population of living cells that are exposed to said serum; and
a molecular species that accelerates induction of death of said cells,
wherein said molecular species is selected from the group consisting of serum albumin, hemoglobin, glycine and glutamic acid.

11. The system according to claim 10, wherein said cells are selected from the group consisting of human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells.

12. The system according to claim 10, wherein said serum comprises fetal bovine serum.

13. A method of preventing cell death in a cell system comprising the steps of:

providing an osmotically unadjusted concentration of a low molecular weight fraction of a serum, said concentration being at least equivalent to a cytotoxic concentration of whole serum sufficient to induce cell death;

providing a molecular species that prevents induction of death of said cells by said whole serum; and

exposing a population of living cells to said fraction and to said molecular species.

14. The method according to claim 13, wherein said cells are selected from the group consisting of human fetal lung fibroblasts, human epithelioid carcinoma cells, and mouse melanoma cells.

15. The system according to claim 13, wherein said fraction comprises a fraction of fetal bovine serum and said whole serum comprises whole fetal bovine serum.

16. The method according to claim 13, wherein said molecular species comprises a thiol or a dithiol homo-dimer thereof.

17. The method according to claim 13, wherein said molecular species comprises at least one of the group consisting of cysteine, glutathione, dithiothreitol, 2-mercaptoethanol, dithionite, thioglycolic acid, DL-homocystine, N-acetyl-L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid), a homo-dimer thereof, and a mixed disulfide thereof.

18. The method according to claim 17, wherein said molecular species has a concentration of between about 1 and 10 mM.

19. A method of preventing serum-induced apoptotic cell death, comprising the steps of:

providing a culture medium, said medium comprising a concentration of a low molecular weight fraction of a serum, said concentration being at least equivalent to a cytotoxic concentration of whole serum sufficient to induce cell death;

25

enriching said medium with an amino acid selected from the group consisting of L-cysteine, L-cystine, and L-tryptophan; and
culturing a cell line in said enriched medium.

20. The method according to claim 19, wherein said amino acid is L-cysteine in a concentration of between about 0.1 mM and 5 mM.

21. The method according to claim 19, wherein said amino acid is L-cystine in a concentration of between about 0.05 mM and 0.5 mM.

22. A method of preventing serum-induced apoptotic cell death, comprising the steps of:

providing a cell culture medium comprising a cytotoxic concentration of whole serum sufficient to induce cell death;

enriching said medium with a substance selected from the group consisting of puromycin hydrochloride, emetine hydrochloride, cycloheximide, actinomycin D, ethidium bromide, L-tryptophan, and D-tryptophan; and
culturing a cell line in said enriched medium.

23. The method according to claim 22, further comprising the step of enriching said medium with a thiol.

24. The method according to claim 23, wherein said thiol is N-acetyl-cysteine.

25. A method of preventing serum-induced apoptotic cell death, comprising the steps of:

providing a cell culture medium comprising a cytotoxic concentration of serum;

culturing a cell line in said medium; and

while said step of culturing is being performed, inhibiting protein synthesis in said cell line.

26. A method of preventing serum-induced apoptotic cell death, comprising the steps of:

providing a cell culture medium comprising a cytotoxic concentration of serum;

culturing a cell line in said medium; and

while said step of culturing is being performed, inhibiting RNA synthesis in said cell line.

27. A method of accelerating serum induced cell death in a cell system comprising the steps of:

providing a medium comprising a cytotoxic concentration of serum, said concentration being sufficient to induce cell death;

exposing a population of living cells to said serum; and

exposing said cells to a molecular species that accelerates induction of death of said cells by said serum, wherein said molecular species is selected from the group consisting of serum albumin, hemoglobin, glycine and glutamic acid.

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FIG. 1

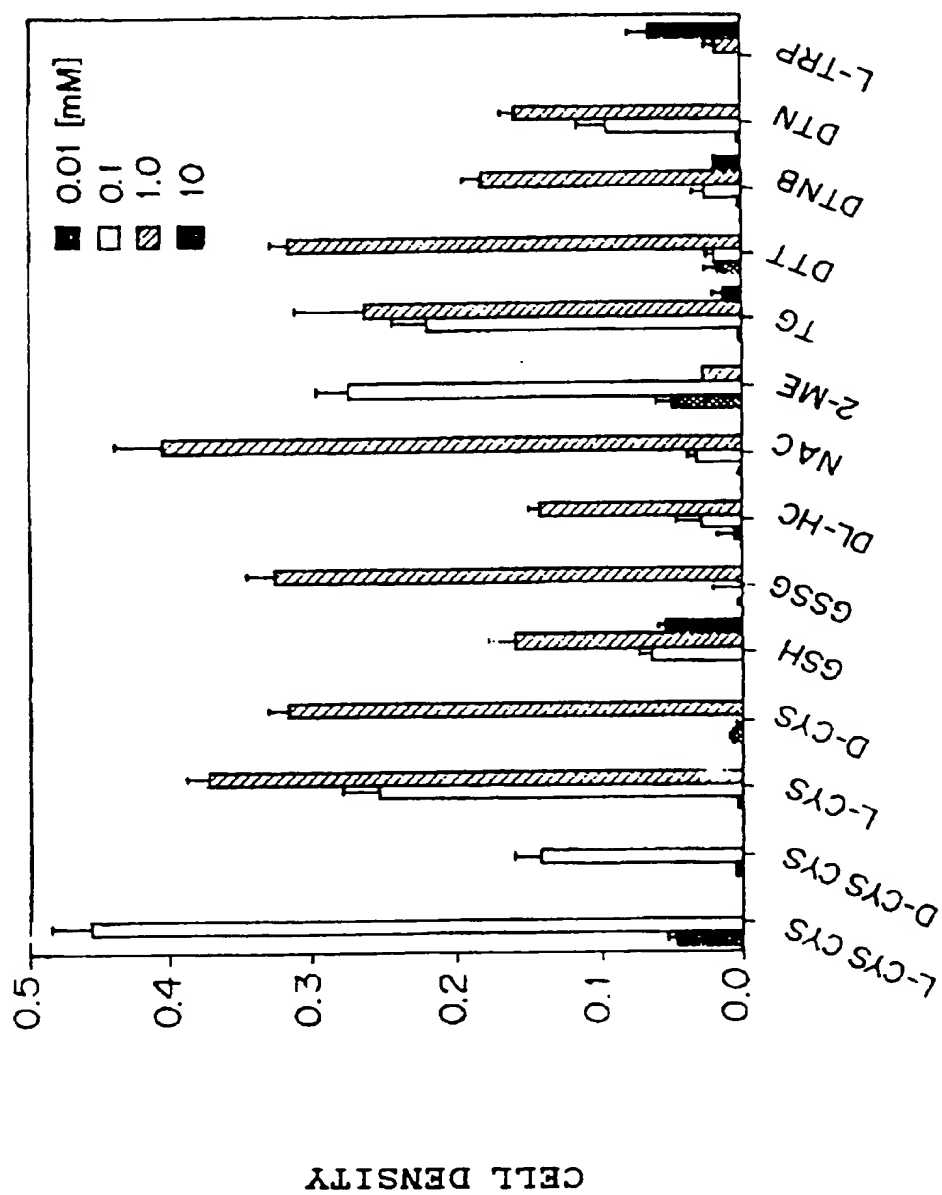
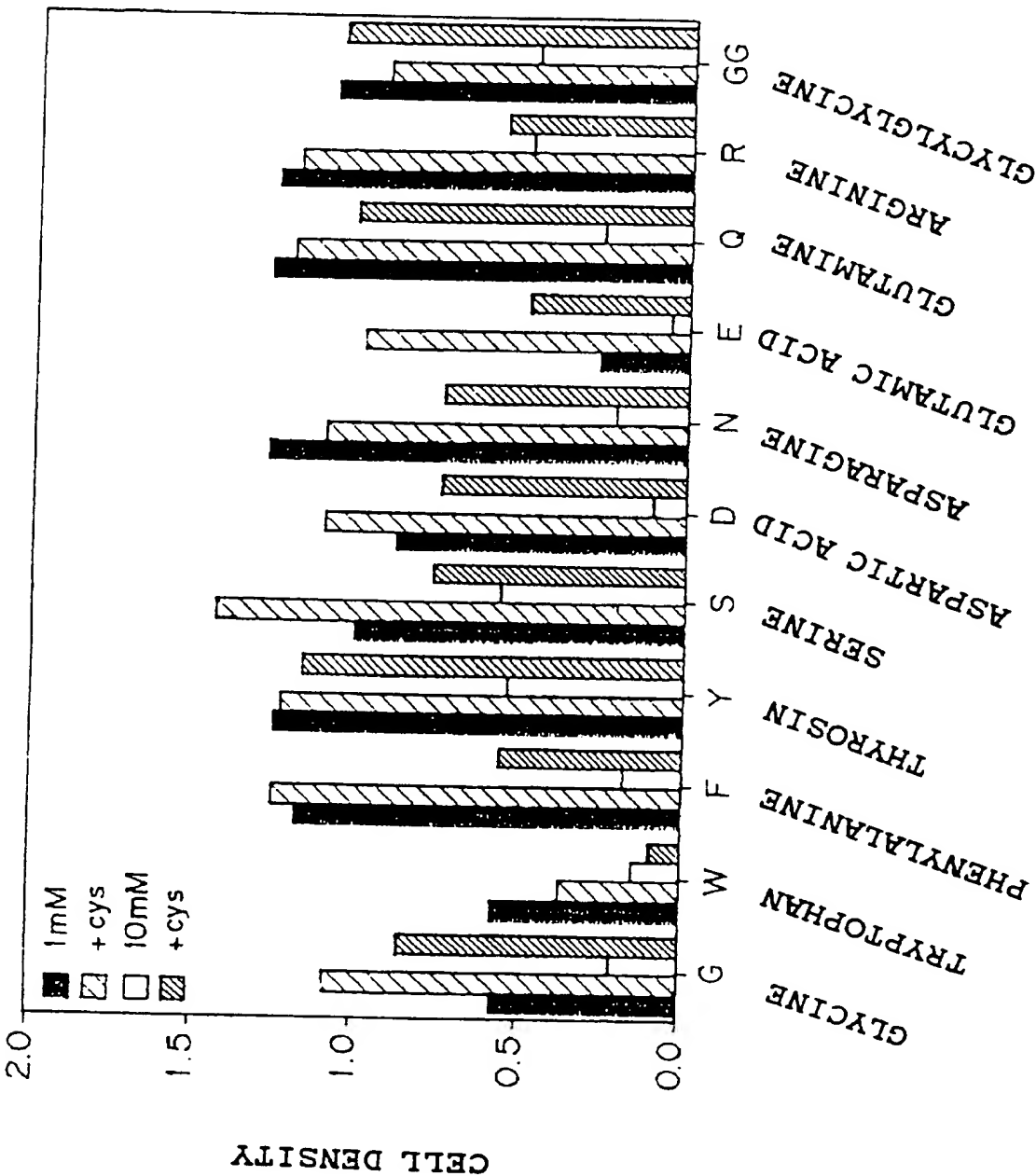


FIG. 2



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FIG. 3

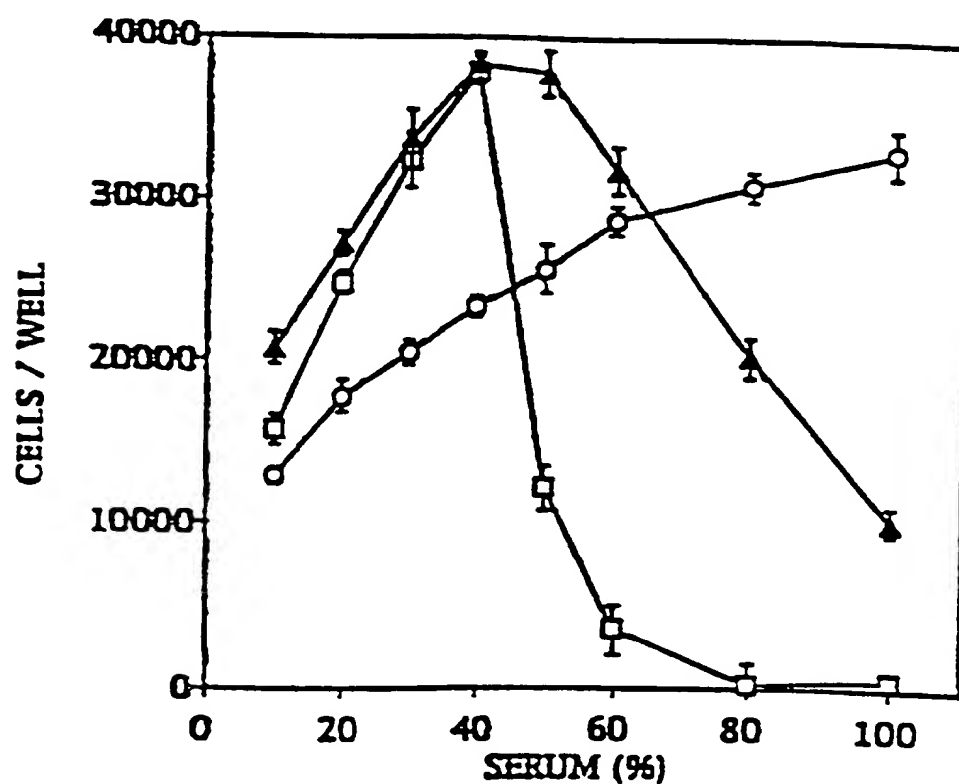
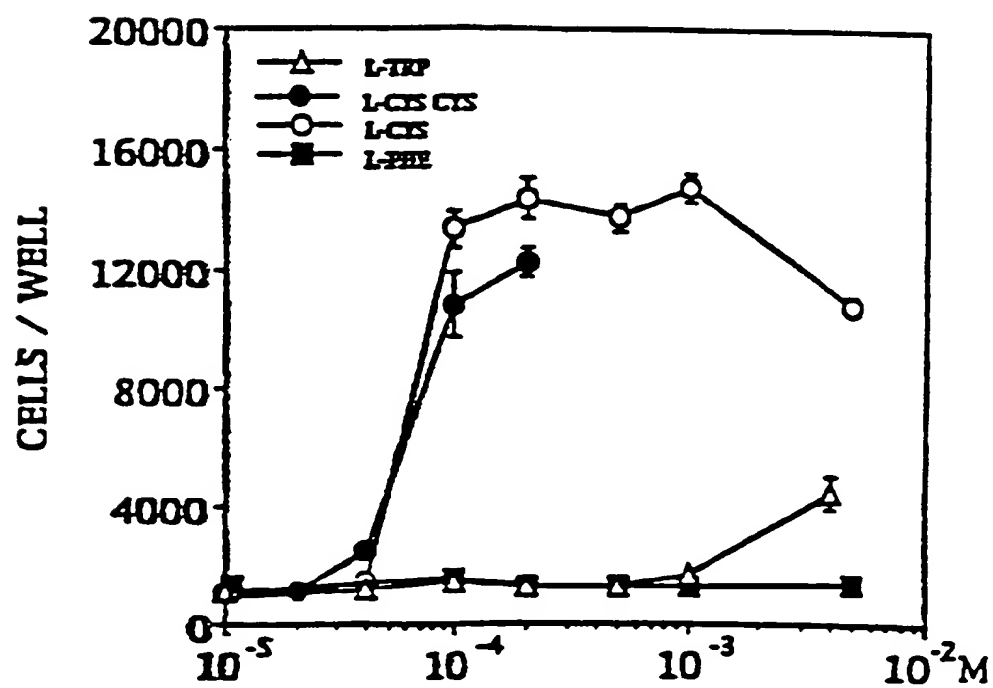
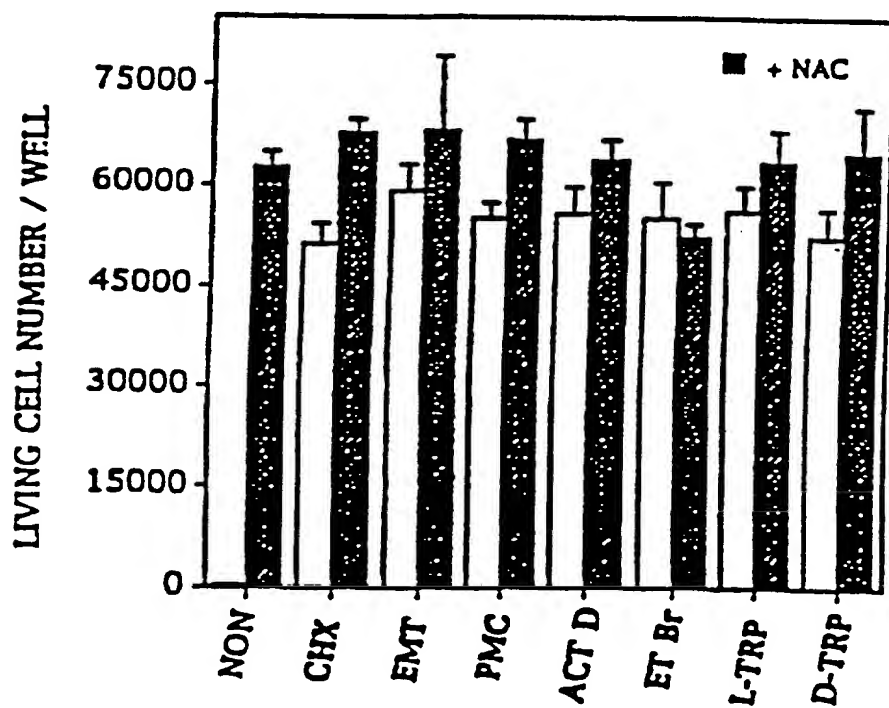


FIG. 4



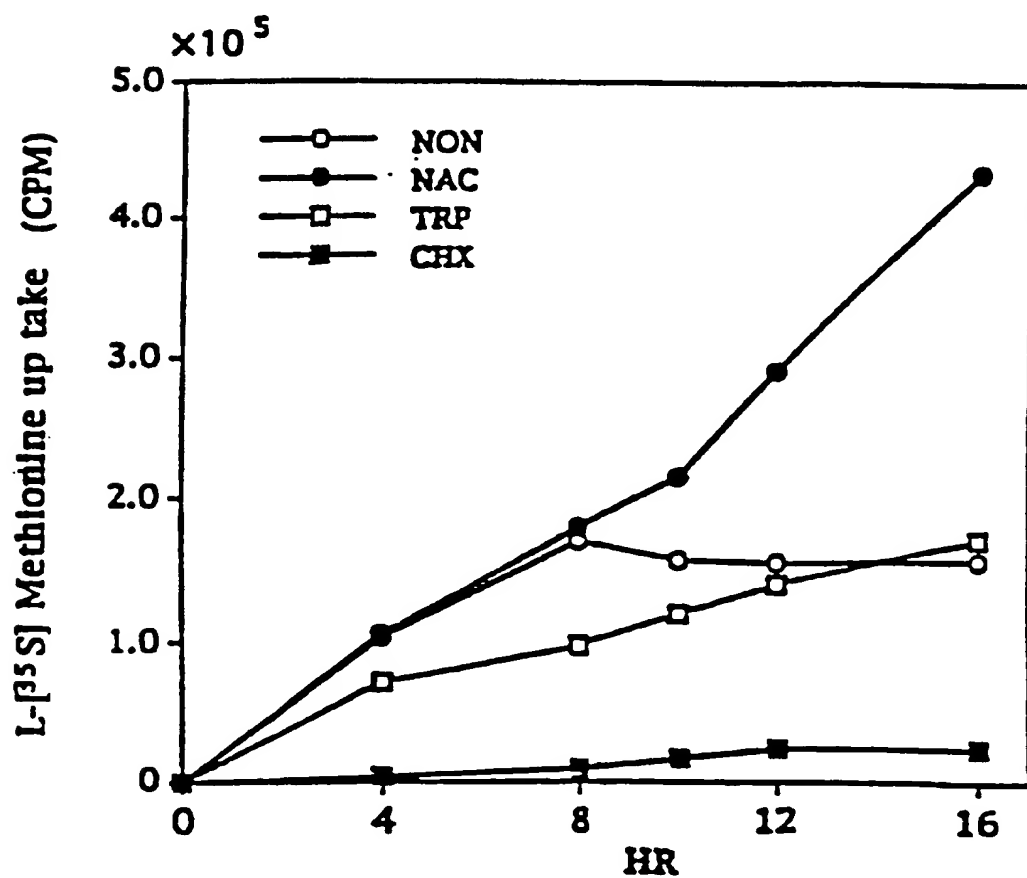
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FIG. 5



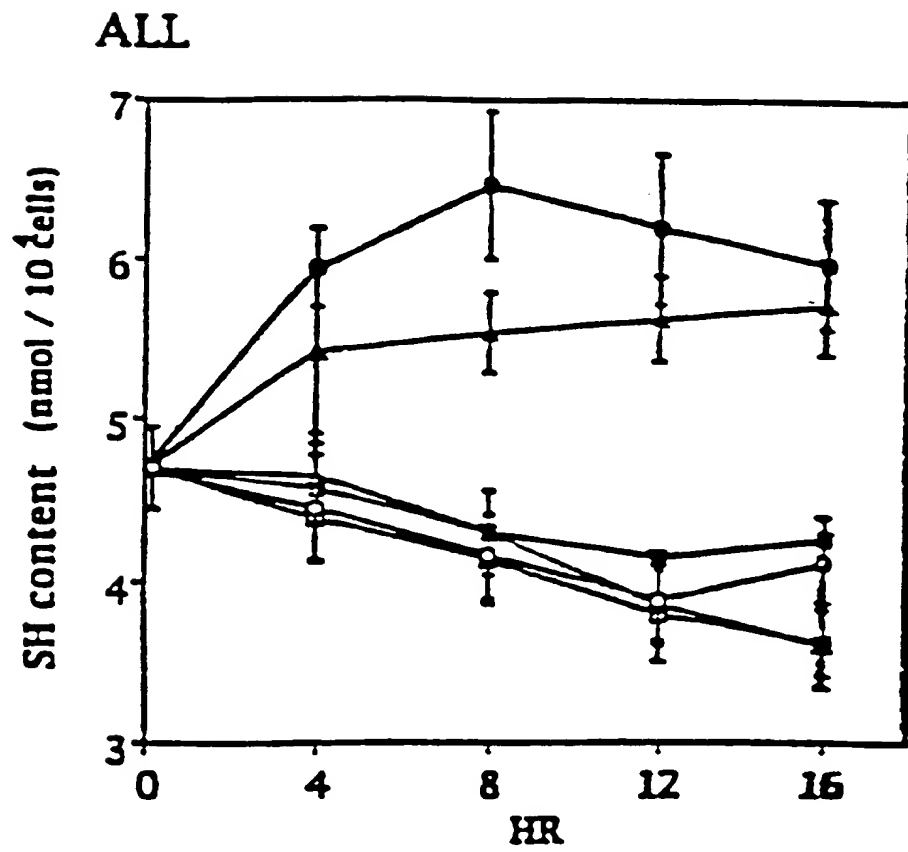
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FIG. 6



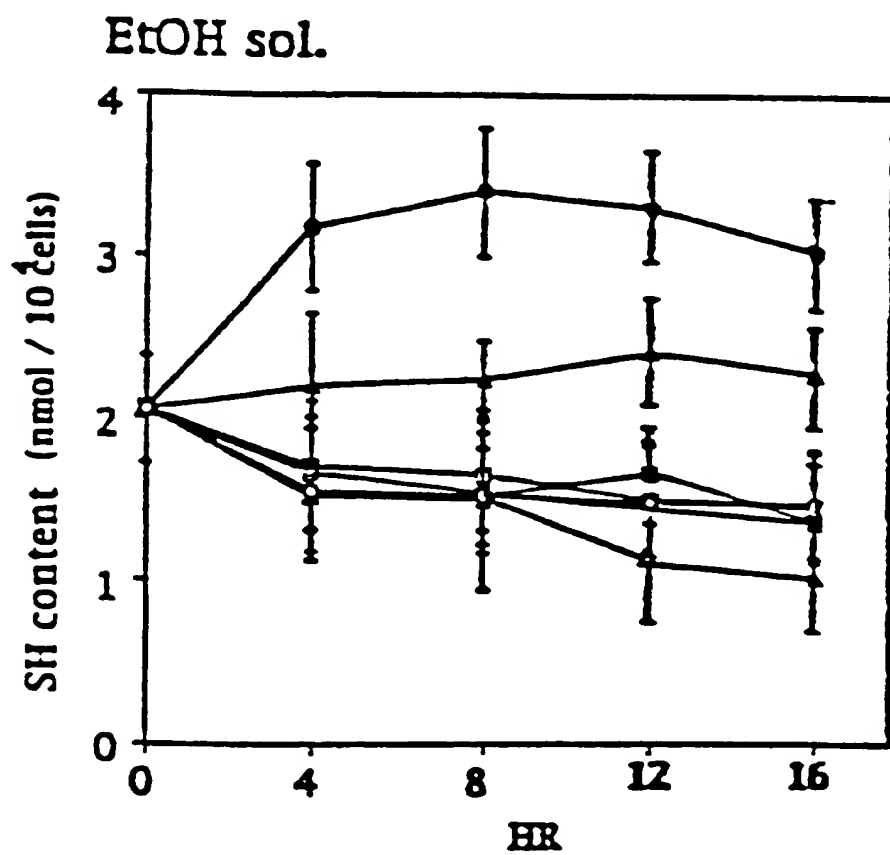
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FIG. 7a



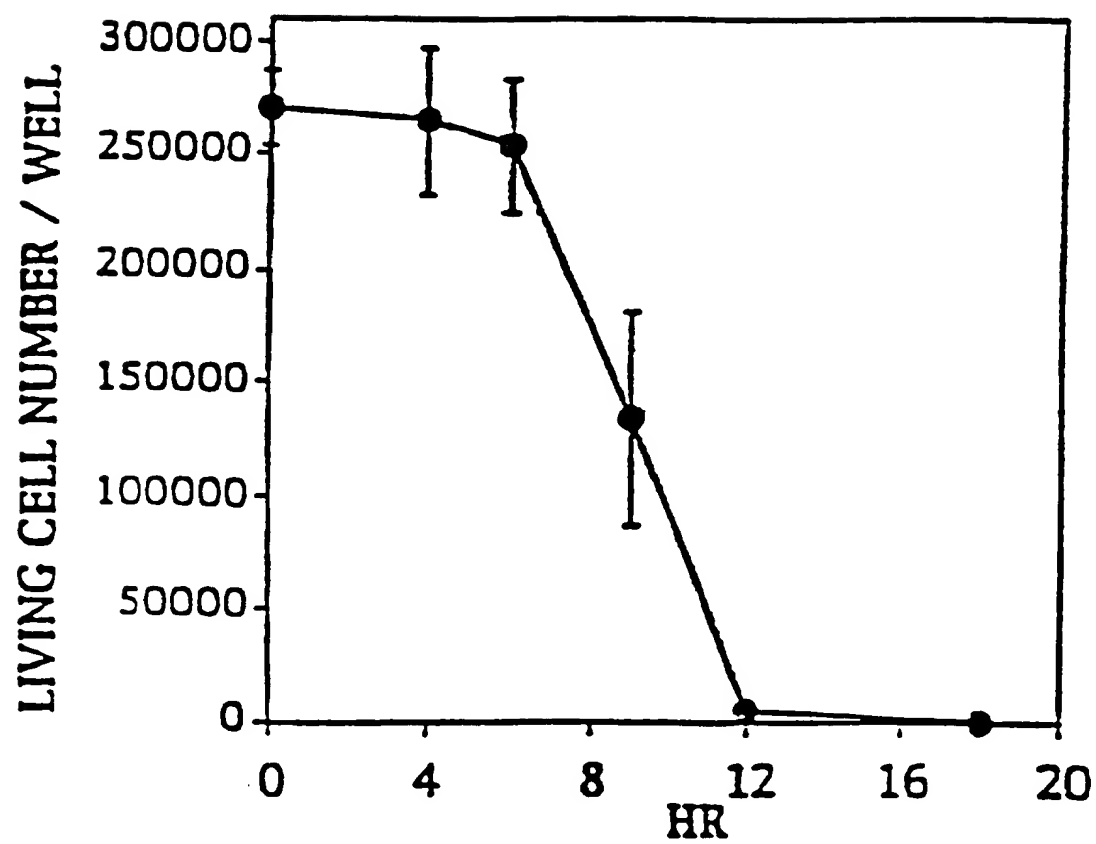
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FIG. 7b



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FIG. 8



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FIG. 9a

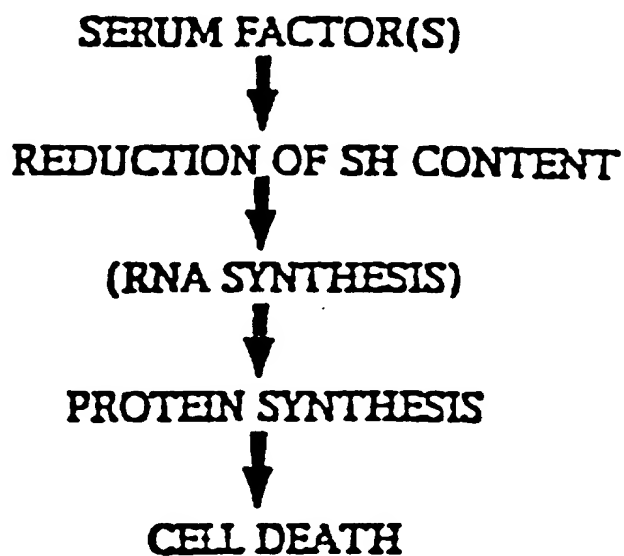
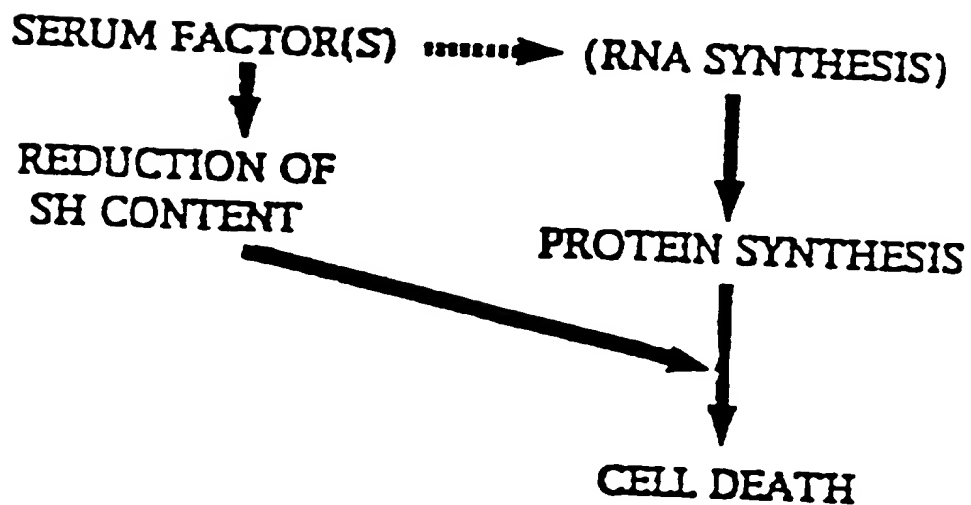


FIG. 9b

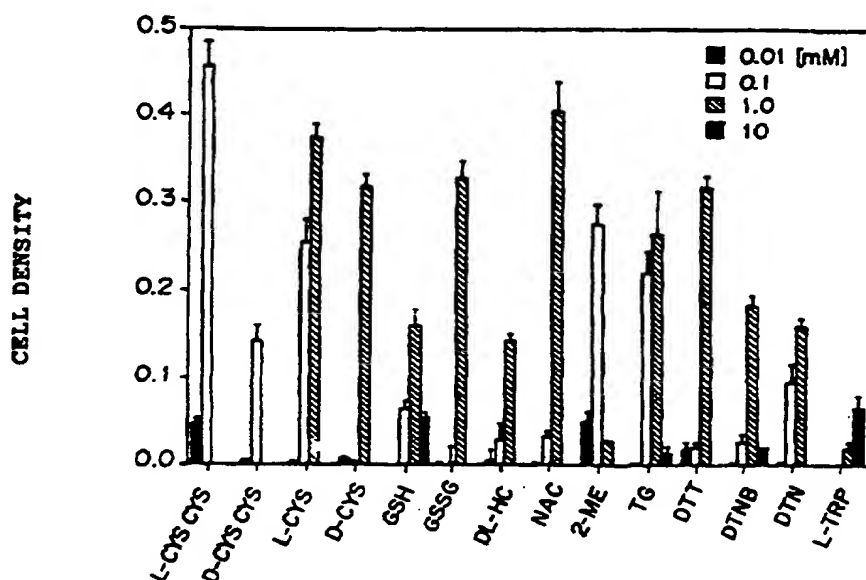




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(71) Applicant: KABUSHIKI KAISHA FRONTIER [JP/JP]; 518, Waseda Tsurumaki-cho, Shinjuku-ku, Tokyo 169 (JP).			
(72) Inventors: NAMIKI, Hideo; Kabushiki Kaisha Frontier, 518, Waseda Tsurumaki-cho, Shinjuku-ku, Tokyo 169 (JP). KU-RITA, Takeshi; 404-5, Fujima, Kawagoe-shi, Saitama-ken 356 (JP).			
(74) Agents: ISHIKAWA, Yasuo et al.; Park Shiba Building, 4F, 17-11, Shiba 2-chome, Minato-ku, Tokyo 105 (JP).			

(54) Title: CELL DEATH ACCELERATOR AND CELL DEATH INHIBITOR



(57) Abstract

A first embodiment of a cell culture system has a cell death accelerator comprising one or more cell death inducing substances, including serum albumin, hemoglobin, glycine and glutamic acid. In a second embodiment a cell death inhibitor comprises one or more kinds of cell death inhibiting substances which include mercapto group containing amino acids, other mercapto group containing compounds and tryptophan. In a third embodiment a cell death inhibitor comprises an inhibitor of RNA or protein synthesis, optionally augmented with a thiol. The system can be applied to selectively induce death of cultured cells, such as neoplastic cell lines, or to inhibit death of other cells, such as neoplastic cell lines or non-neoplastic cells such as brain cells.

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INTERNATIONAL SEARCH REPORT

Inter Application No

PC1/JP 95/01581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O, X	<p>ZOOLOGICAL SCIENCE : SIXTY-FOURTH ANNUAL MEETING OF THE ZOOLOGICAL SOCIETY OF JAPAN, OKINAWA, JAPAN, NOVEMBER 20-23, 1993., vol. 10(supplement), 15 November 1993 HIROSHIMA, JAPAN, page 157 KURITA T; NAMIKI H 'Serum-induced cell death was rescued by thiols' see the abstract</p> <p style="text-align: center;">--- -/--</p>	<p>1-5, 7-9, 13-17, 19-23, 25, 26</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A document member of the same patent family

Date of the actual completion of the international search

23 January 1996

Date of mailing of the international search report

22 February 1996

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/JP 95/01581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 9227 Derwent Publications Ltd., London, GB; Class B04, AN 92-223239 & JP,A,04 148 679 (KURITA T) , 21 May 1992 cited in the application see abstract</p> <p style="text-align: center;">---</p>	<p>1,4-6, 13,16-20</p>
X	<p>ZOOLOGICAL SCIENCE, vol. 10, no. 3, 15 June 1993 HIROSHIMA, JAPAN, pages 431-438, T.KURITA AND H.NAMIKI 'Serum induced cell death' cited in the application see the whole document</p> <p style="text-align: center;">---</p>	<p>10-12,27</p>
A	<p>EP,A,0 295 432 (BEHRINGWERKE AG) 21 December 1988 see the whole document</p> <p style="text-align: center;">---</p>	<p>1-3</p>
P,X	<p>JOURNAL OF CELLULAR PHYSIOLOGY, vol. 161, no. 1, October 1994 pages 63-70, T.KURITA AND H.NAMIKI 'Apoptotic cell death induced by serum and its prevention by thiols' see the whole document</p> <p style="text-align: center;">-----</p>	<p>1-26</p>

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-18, 27
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-18 and 27 in so far as an in vivo method is concerned are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

information on patent family members

International Application No

PL /JP 95/01581

Form PCT/ISA/210 (patent family annex) (July 1992)